

Oral Vaccination of BALB/c Mice with *Salmonella enterica* Serovar Typhimurium Expressing *Pseudomonas aeruginosa* O Antigen Promotes Increased Survival in an Acute Fatal Pneumonia Model

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Pseudomonas aeruginosa is a leading cause of nosocomial pneumonia. We compared the efficacies of oral and intraperitoneal (i.p.) vaccinations of BALB/c mice with attenuated *Salmonella enterica* serovar Typhimurium SL3261 expressing *P. aeruginosa* serogroup O11 O antigen to protect against *P. aeruginosa* infection in an acute fatal pneumonia model. Oral and i.p. vaccines elicited O11-specific serum immunoglobulin G (IgG) antibodies, but IgA was observed only after oral immunization. Challenge of orally vaccinated mice with an O11 strain (9882-80) at 6 and 12 times the 50% lethal dose showed increased survival in mice that received the vaccine compared to phosphate-buffered saline (PBS)- and vector-treated controls; no difference in survival was seen with a heterologous strain, 6294 (serogroup O6). In addition, significant protection against 9882-80 was not observed in i.p. vaccinated animals. Bronchoalveolar lavage fluid taken from immunized mice harbored O11-specific IgA and IgG in orally immunized mice but only modest levels of IgG in i.p. vaccinated mice. To correlate protection, opsonophagocytosis assays were performed with pooled sera from orally immunized animals. Efficient killing of five O11 clinical isolates was observed, while no killing was noted with 6294, indicating that the recombinant SL3261 oral vaccine induces an O11-specific reaction. We next determined the ability of orally vaccinated animals to clear bacteria from their lungs. Following *P. aeruginosa* challenge, the numbers of viable bacteria were significantly fewer in orally vaccinated animals than in PBS- and vector-treated controls. Our results suggest that oral immunization with recombinant SL3261 is efficacious in protection against pneumonia caused by *P. aeruginosa*.

Pseudomonas aeruginosa is an environmentally ubiquitous organism and a leading cause of morbidity and mortality in hospital-acquired pneumonia (29). Susceptible individuals, usually those who are critically ill (54) or immunosuppressed (16), are initially colonized in the upper respiratory tract by serum-resistant lipopolysaccharide (LPS) smooth strains (containing long O-antigen repeating units), which are distinct from the serum-sensitive LPS rough strains (lacking O antigen) found in chronically infected cystic fibrosis patients (23). Particularly alarming is the mortality associated with pneumonia caused by *P. aeruginosa* in ventilator-dependent patients, with rates exceeding 40% (4, 17). Exacerbating this problem is the increasing emergence of multiple-antibiotic-resistant strains of *P. aeruginosa* (19), likely due to overexposure to antibiotics (28, 34). In addition, *P. aeruginosa* is naturally resistant to many antibiotics because of low outer membrane permeability and the existence of drug efflux components in the cytoplasmic membrane (49, 50). Together, these characteristics give rise to difficulties in treating *P. aeruginosa* infections and thus warrant the need for a vaccine product that could be used for producing active or passive therapeutic agents. *P. aeruginosa* vaccine candidates include outer membrane proteins (9, 21, 33, 37, 61), cytosolic proteins (60), extracellular proteins, such as those of

flagella (15, 39) and pili (3, 57, 58), and extracellular polysaccharides, such as alginate (59) and LPS (11, 12).

The O-antigen portion of LPS is the principal target of the immune response. Twenty serogroups (36), including many subgroups (31), of *P. aeruginosa* have been identified based on differences in saccharide composition and structure of the O antigen. This complexity is the basis for problems associated with developing LPS vaccines. Usually protection is elicited against the strain from which the LPS formulation was constructed (10, 12, 46, 47), with minimal opsonic reactivity to subgroup strains within the same serogroup (25, 27). This dilemma may be remedied by presentation of the O antigen from a selected organism in the context of a live attenuated organism. Attenuated vaccine strains have been shown to be advantageous based on their mimicking of natural infection pathways, thus leading to enhanced immunogenicity. Recent work by Priebe et al. demonstrated that a live attenuated *aroA* deletion mutant of *P. aeruginosa* O2/O5 elicits high levels of opsonic anti-LPS titers (52) and protection against multiple serogroup O2/O5 strains in mice (53). In the former study, opsonic killing activity was not abolished after antiserum adsorption to *P. aeruginosa* O-antigen-deficient strains, suggesting that the protective response was specific to the LPS (52).

Over the last decade, the use of live attenuated *Salmonella* strains for heterologous antigen delivery has increased considerably. The advantage of oral delivery of these strains is their ability to activate systemic as well as local and distant mucosal compartments of the immune system (30, 55). Work per-

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TABLE 1. Bacterial strains used in this study

Strain	Description	Reference(s)
<i>Salmonella</i>		
SL3261/pLAFR1	Δ aroA derivative of SL1344 containing a control cosmid vector	45
SL3261/pLPS2	Δ aroA derivative of SL1344 harboring a plasmid containing the PA103 serogroup O11 O-antigen gene cluster	45
<i>P. aeruginosa</i>		
PA103	Wild-type strain; serogroup O11	35
PA103 (<i>galU</i>)	O-antigen-deficient serogroup O11 derivative	13
9882-80	Clinical isolate; serogroup O11	45
6073	Clinical isolate; serogroup O11	18, 51
6077	Clinical isolate; serogroup O11	18, 51
6206	Clinical isolate; serogroup O11	18, 51
6294	Clinical isolate; serogroup O6	18, 51

formed by various investigators showed that oral immunization with attenuated *Salmonella enterica* serovar Typhimurium SL3261 expressing *P. aeruginosa* O11 O antigen from plasmid pLPS2 (SL3261/pLPS2) (22) facilitated clearance in the gastrointestinal tract (GI) after oral challenge with a *P. aeruginosa* O11 strain (45). Decreased bacterial load in the GI tract was also observed after intraperitoneal (i.p.) immunization with high-molecular-weight O-polysaccharide antigen, which was shown to stimulate only circulating immunoglobulin G (IgG) and IgM antibodies. These results suggest that systemic vaccination is sufficient to protect against bacterial colonization of mucosal surfaces in the gut colonization model (45). In the present study, we compared the efficacies of oral and i.p. vaccinations with SL3261/pLPS2 to protect against acute fatal pneumonia caused by *P. aeruginosa*. We present data suggesting that mucosal rather than systemic vaccination is more efficacious in this model.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains and plasmids are listed in Table 1 along with their descriptions and sources.

Preparation of bacterial strains for vaccination. The serogroup O11 gene locus is contained within cosmid vector pLAFR1, referred to here as pLPS2 (22). Constructs SL3261/pLAFR1 and SL3261/pLPS2 are referred to here as the vector and vaccine strains, respectively. *Salmonella* vector and vaccine strains were inoculated into Luria-Bertani broth containing tetracycline at 10 μ g/ml and grown to an optical density at 650 nm (OD_{650}) of 0.5. Cells were harvested by centrifugation at $3,100 \times g$ for 20 min at 4°C and resuspended in phosphate-buffered saline (PBS) for i.p. vaccination or PBS supplemented with 3% sodium bicarbonate and 2% sucrose for oral vaccination. Prior to administration to animals, bacterial cells were adjusted spectrophotometrically and plated on tryptic soy agar (TSA) to determine viable cell counts.

Oral and i.p. vaccinations. The University of Virginia Animal Care and Use Committee approved all procedures used in this work. Six- to 8-week-old BALB/c mice (Jackson Laboratories, Bar Harbor, Maine, or Harlan Sprague-Dawley Farms, Chicago, Ill.) were housed under pathogen-free conditions and fed autoclaved rodent feed and acid-free water. For oral vaccination, mice were fed 100 μ l of either PBS or the *Salmonella* vector and vaccine strains (1×10^9 to 5×10^9 CFU) by intragastric gavage with a 20/25-mm feeding needle (Popper and Sons, Inc., New Hyde Park, N.Y.) attached to a 1.0-ml Becton Dickinson (BD) Luer-Lock syringe (Fisher Scientific, Pittsburgh, Pa.). Oral inoculation was repeated once per week for a total of 4 weeks. For i.p. vaccination, mice were inoculated with a single dose of either PBS or each *Salmonella* strain (10^6 CFU) by i.p. administration with a 28.5-gauge BD insulin syringe.

Survival studies. Challenge experiments were performed with *P. aeruginosa* strains 9882-80 (serogroup O11) and 6294 (serogroup O6). The appropriate strains were inoculated onto TSA and grown for a maximum of 12 h at 37°C. Cells were resuspended in PBS to an OD_{650} of 0.5 and diluted to the appropriate concentration to obtain the desired challenge dose in 20 μ l. Prior to challenge,

mice were anesthetized by i.p. injection of 0.2 ml of freshly prepared filter-sterilized ketamine (6.7 mg/ml) and xylazine (1.3 mg/ml) in 0.9% saline. Once the mice were anesthetized, 10 μ l of the bacterial inoculum was placed into each nostril (total of 20 μ l/mouse). The mice were observed carefully for morbidity and mortality for up to 1 week.

Collection of BAL fluid. Mice were euthanized by anesthetic overdosing with 0.5 ml of freshly prepared filter-sterilized ketamine and xylazine injected i.p. The tracheas of these animals were exposed by standard procedures and cannulated with a 20-gauge, 1.16-in., 1.1- by 30-mm BD tracheal cannula attached to a Luer-Lock syringe needle. One milliliter of PBS was introduced into the lungs via the tracheal cannula and carefully extracted. This procedure was repeated once more, for a total of 2 ml of PBS for each lavage. The bronchoalveolar lavage (BAL) fluid was analyzed immediately by an indirect enzyme-linked immunosorbent assay (ELISA) for *Pseudomonas*-specific antibodies.

Serum collection. Blood samples were collected from the tail vein of each mouse after warming with a heat lamp. The samples were allowed to stand at room temperature for 4 h and then were incubated overnight at 4°C. Serum was collected by centrifugation at $1,700 \times g$ for 10 min and then was stored at -80°C until use. All serum samples were diluted in PBS supplemented with 1% bovine serum albumin (PBS-B) prior to use in ELISAs.

Indirect and competition ELISAs. The optimal concentrations of whole organisms (either *P. aeruginosa* or SL3261) for use as coating antigens were determined by criss-cross serial dilution with *P. aeruginosa* serogroup O11 (Accurate Chemical, Westbury, N.Y.)- or *Salmonella* O antiserum factor 4 (Difco Laboratories, Detroit, Mich.)-specific polyclonal antibodies. *P. aeruginosa* organisms or SL3261 were inoculated onto TSA and grown for a maximum of 12 h at 37°C. Bacteria were suspended in 0.1 M sodium phosphate buffer to an OD_{650} of 0.5, diluted 1:2, and used to coat Immulon 2 HB ELISA plates (Thermo Labsystems, Franklin, Mass.). The plates were incubated overnight at 4°C to allow for sufficient antigen coating, washed with PBS supplemented with 0.05% Tween 20 (PBS-T), and blocked by 1 h of incubation at room temperature with PBS-B. After a second wash with PBS-T, the ELISA plates were stored at 4°C until use.

For antibody titer determinations, serum and BAL samples were serially diluted in PBS-B, and 100 μ l was placed into each well on antigen-coated plates in duplicate. After overnight incubation at 4°C, the plates were washed three times with PBS-T and dried. Secondary antibodies (anti-mouse total IgG, IgG1, IgG2a, IgG2b, IgG3, or IgA conjugated to alkaline phosphatase [Southern Biotechnology Associates, Inc., Birmingham, Ala.]) were diluted 1:5,000 in PBS-B, and 100 μ l was added to appropriate wells and incubated at 37°C for 90 min. After incubation, the plates were carefully washed again three times with PBS-T and dried. Next, 200 μ l of the substrate, consisting of 4-nitrophenyl phosphate disodium salt hexahydrate (PNPP) (Sigma Chemical Co., St. Louis, Mo.) diluted to 1 mg/ml in PNPP substrate solution (10% diethanolamine, 25 μ M MgCl_2), was added to each well and incubated for 30 min in darkness at room temperature. PNPP hydrolysis was terminated by the addition of 50 μ l of 3 M NaOH to each well, the plates were examined at OD_{405} by use of a Molecular Devices Thermo microplate reader, and the data were displayed by use of SOFTmax Pro version 1.1 software.

For IgG subtype quantification, a mouse immunoglobulin standard panel (100 μ g/ml; Southern Biotechnology Associates) was serially diluted and used to coat ELISA plates. The appropriate secondary antibody was used, and the ELISA was carried out as described above. IgG subtype quantification for serum samples was

based on standard curves that were designed for each antibody isotype by use of GraphPad (San Diego, Calif.) Prism version 4 software.

For the competition ELISA, plates coated with PA103 whole organisms were used. The ELISA was performed as described above but with the following exceptions. To a single constant 1:5,000 dilution of pooled sera from vaccine-inoculated mice were added increasing amounts (0 to 5,000 ng) of free purified serogroup O11 LPS (26). The competition ELISA was performed twice in triplicate.

SDS-PAGE and Western immunoblotting. Whole-cell lysates of *P. aeruginosa* and *Salmonella* organisms were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) according to the standard procedure described by Laemmli (32) with a Novex X-Cell Surelock minicell system (Invitrogen, Carlsbad, Calif.). Tris-bis-polyacrylamide gels (12.5%) were cast in 1.0-mm Invitrogen cassettes. After PAGE separation was completed, lysates were electroblotted onto Trans-Blot 0.2- μ m-pore-size pure nitrocellulose membranes (Bio-Rad Laboratories, Hercules, Calif.) by use of a Bio-Rad Mini Trans-Blot electrophoretic transfer cell. Membranes were blocked and then probed with *Pseudomonas* serogroup O11- or *Salmonella* O antiserum factor 4-specific polyclonal antibodies, followed by incubation with anti-rabbit secondary antibodies conjugated to alkaline phosphatase (Sigma). Reactions were visualized by the addition of Sigma fast 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium.

Opsonophagocytosis assay. The opsonophagocytosis assay was performed in this work as previously described (44) with minor modifications. Briefly, the assay consists of four components: freshly prepared dextran-purified white blood cells (obtained from 30 ml of human blood by use of an 18-gauge, 1-in. hypodermic needle [SIMS Portex Inc., Keene, N.H.] attached to a 30-ml BD syringe), baby rabbit serum as a complement source (Accurate Chemical), pre- and postimmunization sera at various dilutions, and the target *P. aeruginosa* strains. Prior to use in this study, all target strains were confirmed for their ability to resist serum killing by repeated incubations with normal human serum and passages in Luria-Bertani broth. All four components were incubated together at 37°C with slight agitation for 90 min, followed by the determination of viable cell counts on TSA. The percentage of killing was determined by comparing the number of colonies isolated from diluted postimmunization antisera to the number of colonies obtained from preimmunization sera at the same dilution. Under these conditions, observation of 50% killing is considered to be biologically significant and thus serves as the point at which antisera are considered positive for opsonic killing activity.

Statistical analyses. All analyses were performed by use of GraphPad Prism version 4 software. ELISA endpoint titers were calculated by linear regression of duplicate measurements of adjusted OD₄₀₅s and were expressed as the reciprocal dilution. OD₄₀₅s of preimmunized samples at the same dilution were subtracted from those of postimmunization samples. The x intercept served as the endpoint titer. *Pseudomonas*-specific total IgG and IgA titers were compared by using the Kruskal-Wallis U test for analysis of three groups or the Mann-Whitney U test for comparison of two groups. Oral IgG subtype values were analyzed by use of a mixed-linear-model methodology. The natural logarithms of the IgG subtype values were modeled by use of a compound symmetric covariance structure to adjust for correlation among measurements obtained from the same mouse. For survival studies, data are presented as Kaplan-Meier survival curves and were analyzed by the log-rank test. For comparison of the numbers of viable bacteria obtained in lung homogenates from PBS-, vector-, and vaccine-inoculated animals, the data were transformed to natural logarithms and analyzed by one-way analysis of variance.

RESULTS

Immunogenicity of the *P. aeruginosa* serogroup O11 O antigen expressed from *S. enterica* serovar Typhimurium SL3261 after oral and i.p. vaccinations. Goldberg et al. previously identified the expression of the PA103 serogroup O11 O antigen in SL3261 (22), and Pier et al. showed that this organism is nonvirulent in BALB/c mice (45). To study the protective efficacy afforded by the vaccine strain against acute pneumonia caused by *P. aeruginosa* in mice, animals were vaccinated by orogastric gavage or i.p. injection. The kinetics of the *Pseudomonas*- and *Salmonella*-specific serum IgG and IgA antibody responses to whole bacterial cells in vaccine-inoculated mice were monitored and compared to those in PBS- and vector-

treated mice (data not shown). Serum samples taken 1 week after each booster showed an increase in the levels of *Pseudomonas*-specific IgG and IgA in orally vaccinated animals; the highest total IgG and IgA titers were observed 1 week after the final booster (Fig. 1A). The same trend was observed in i.p. vaccinated mice; animals receiving the vaccine showed significant *Pseudomonas*-specific serum total IgG titers compared to the controls (Fig. 1B). Interestingly, serum from i.p. vaccinated mice had *Pseudomonas*-specific total IgG titers that were significantly (approximately twofold) higher than those seen in orally vaccinated mice (Fig. 1A and B). *Pseudomonas*-specific serum IgA was not detected above preimmune serum levels in i.p. vaccinated animals. Pooled sera from orally vaccinated animals were reactive to four additional serogroup O11 strains in an ELISA, with total IgG titers of >9,000; the highest titer was seen for the parental O-antigen strain, PA103 (>11,500) (Fig. 1C). Similar specificity was observed with pooled sera from i.p. vaccinated mice, which showed high serum total IgG titers for all serogroup O11 strains tested (>11,000 to 25,000); the highest titer was again seen for strain PA103 (Fig. 1C). IgG cross-reactivity was observed for a heterologous serogroup O6 *P. aeruginosa* strain, 6294, when ELISA plates coated with whole organisms and antisera from both orally and i.p. vaccinated animals were used (titer, <4,500). These results suggest that shared antigens exist among *Salmonella* and *Pseudomonas* but that the cross-reactivity of these antigens is below that observed for LPS-specific antigens (data not shown). Serum IgA reactivity in pooled sera was seen only in orally vaccinated animals with titers exceeding 5,000 (data not shown).

The IgG subtype responses were determined for both orally and i.p. vaccinated animals. Oral vaccination failed to induce statistically different levels of serum IgG2a and IgG3 antibodies ($P = 0.21$). The levels of both serum IgG2a and IgG3 antibodies were significantly higher than those of either IgG1 or IgG2b (Fig. 1D). In contrast, i.p. vaccination induced higher levels of IgG3 than of all the other IgG subtypes; these levels were approximately fourfold higher than the levels of IgG3 in orally inoculated mice (Fig. 1D). In addition, the levels of IgG2a antibodies in i.p. vaccinated animals were higher than those of IgG1 and IgG2b and were approximately twofold higher than the levels of IgG2a seen after oral vaccination. These results suggest that i.p. inoculation with the vaccine strain induces higher levels of *Pseudomonas*-specific serum IgG antibodies, with a distinct ratio of IgG subclasses, than does oral immunization.

To determine the reactivity induced by the vaccine, whole-cell lysates were analyzed by Western immunoblotting with pooled sera from orally vaccinated mice (Fig. 2A). Mouse antisera reacted with all five wild-type O11 strains and with the *Salmonella* organisms. No reactivity to 6294 (serogroup O6) or the O-antigen-deficient PA103 (*galU*) mutant was seen (13), suggesting that the immune response in these mice is directed primarily to the homologous (*Salmonella*) and heterologous (*P. aeruginosa*) O antigens.

To confirm the *P. aeruginosa* serogroup O11 O-antigen specificity of this reaction, a competition ELISA with purified *P. aeruginosa* serogroup O11 LPS was performed. Although precise antibody affinity was not determined due to the heterogeneity of LPS molecules, increasing amounts of free O11 LPS

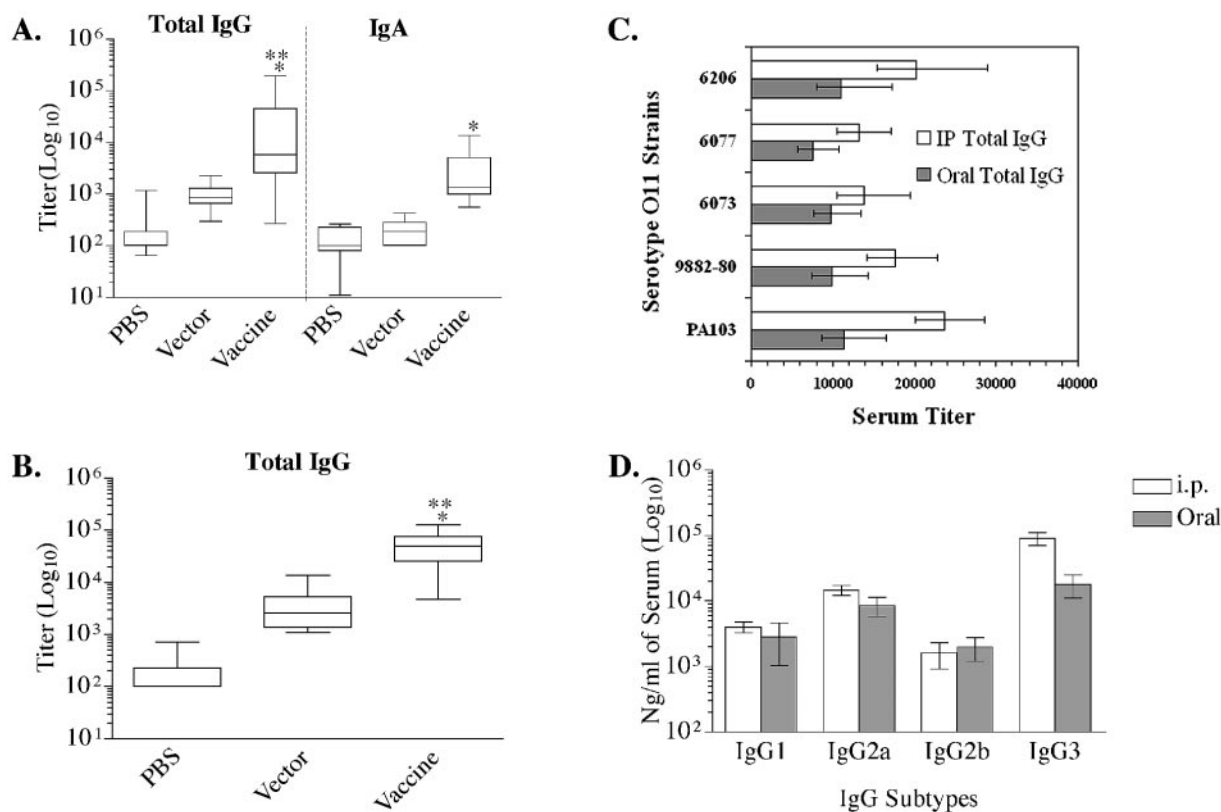


FIG. 1. Complete antibody titers for PBS- and SL3261-vaccinated mice after oral and i.p. vaccinations. (A) Serum *P. aeruginosa* PA103-specific (O11) total IgG and IgA from orally vaccinated BALB/c mice. (B) PA103-specific total IgG from i.p. vaccinated animals. (C) Total IgG to heterologous serogroup O11 strains. (D) IgG subtype responses in orally and i.p. inoculated animals, as determined by an ELISA on plates coated with *Pseudomonas* whole organisms. (A and B) Box and whisker plots show endpoint titers in log₁₀ units as determined by linear regression. The box is marked by the median and depicts the first and third quartiles, while the whiskers depict the range. Significant differences in the titers of *Pseudomonas*-specific antibodies were observed for orally vaccinated mice in panel A (single asterisk) (*P* value determined by Kruskal-Wallis analysis for IgG and IgA: <0.0001; *P* values determined by pairwise Mann-Whitney analysis for IgG and IgA: for PBS versus vaccine, <0.0001, and for vector versus vaccine, <0.0001) and for i.p. vaccinated mice in panel B (single asterisk) (*P* value determined by Kruskal-Wallis analysis: <0.0001; *P* values determined by pairwise Mann-Whitney analysis: for PBS versus vaccine, <0.0001, and for vector versus vaccine, <0.0001). i.p. vaccination with the vaccine strain induced significantly higher levels of serum total IgG than did the orally delivered vaccine strain (double asterisks) (*P* value determined by Mann-Whitney analysis: 0.0034). (C) Endpoint titers were determined by linear regression for pooled sera derived from animals receiving oral or i.p. inoculations. Error bars indicate 95% confidence intervals. (D) Level of each IgG subtype calculated from standard curves that were generated for secondary antibody reactivity to each IgG subtype. Error bars indicate standard deviations.

led to a decline in the percent binding of serogroup O11-specific antibodies from vaccinated mice (Fig. 2B).

Oral immunization provides increased survival of infection with *P. aeruginosa* strain 9882-80 (O11) in an acute fatal pneumonia model. To compare the abilities of the vaccine strain to confer protection against acute pneumonia caused by *P. aeruginosa* in orally and i.p. vaccinated animals, mice were challenged by intranasal infection with noncytotoxic *P. aeruginosa* serogroup O11 strain 9882-80. In the first survival study, anesthetized orally vaccinated mice were challenged with 1.12×10^8 CFU, approximately six times the 50% lethal dose (LD₅₀) calculated for this strain. A significant increase in survival, as a function of time postinfection, was observed in mice that received the recombinant vaccine compared to mice that received either PBS or vector alone (Fig. 3A). In the second survival study, orally vaccinated mice were challenged intranasally with 2.34×10^8 CFU, approximately 12 times the LD₅₀ of 9882-80. A significant level of survival was again observed in mice that received the vaccine strain compared to mice that

received the vector alone (Fig. 3B). Interestingly, intranasal challenge of i.p. vaccinated mice with 9882-80 (1.13×10^8 CFU) failed to provide a difference in survival between vaccine-treated mice and PBS- and vector-treated mice (controls) (Fig. 3C), even though the levels of serum IgG antibodies seen in these mice were significantly higher than those seen in orally vaccinated mice (Fig. 1A to D).

To determine whether protection could be extended to heterologous *P. aeruginosa* strains and to examine whether cross-reactive epitopes contributed to protection in this model, orally vaccinated mice were infected with strain 6294 (serogroup O6) at a challenge dose approximately six times the LD₅₀ calculated for this strain. No difference in survival between vector- and vaccine-inoculated mice was noted, supporting our earlier finding that the vaccine strain induces a serogroup O11-specific response and suggesting that cross-reactive epitopes are not protective (*P* value determined by log-rank analysis: 0.0849); median survival times were 56.5 h with the vector and 26.5 h with the vaccine (data not shown). Altogether, these results

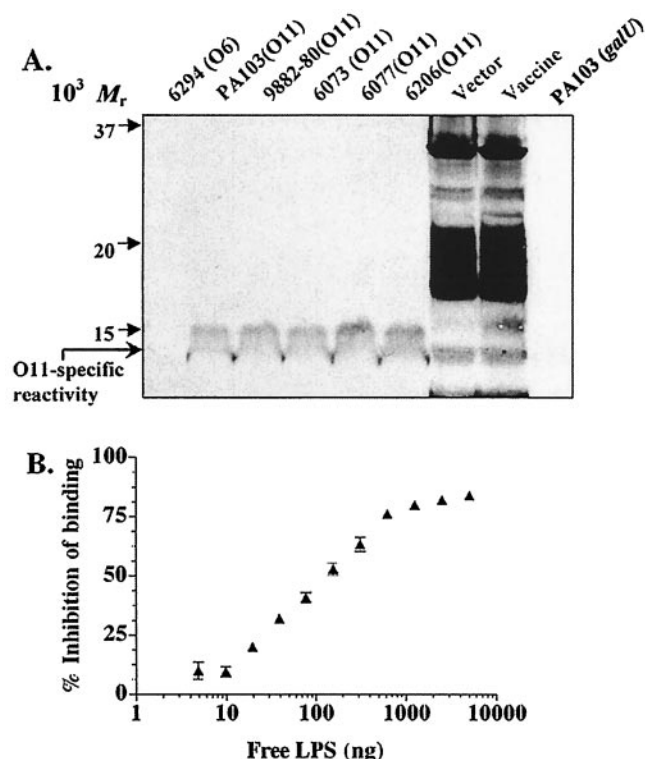


FIG. 2. Reactivity of serum from orally vaccinated mice. (A) Western immunoblot analysis of whole-cell lysates from five heterologous serogroup O11 strains, a serogroup O6 strain, and an O-antigen-deficient mutant of PA103 (*galU*) (13) with pooled sera from orally vaccinated animals. (B) Competition ELISA with diluted pooled sera from orally vaccinated mice (1:5,000) and increasing amounts of free serogroup O11 LPS on ELISA plates coated with PA103 whole organisms. Data were analyzed by nonlinear regression and are represented as percent inhibition by antisera from vaccine-inoculated mice. Error bars indicate standard deviations.

suggest that the vaccine strain induces serogroup O11-specific protection and is more efficacious when delivered orally.

Identification of *P. aeruginosa*-specific antibodies in BAL fluid from orally and i.p. vaccinated mice. With evidence indicating that oral rather than i.p. immunization provides increased survival in the *P. aeruginosa* pneumonia model, we wanted to determine whether a difference in the antibody profiles existed at the site of infection after either vaccination. To accomplish this, BAL was performed on mice after oral and i.p. inoculations. In mice that received the oral vaccine, high levels of total IgG and IgA in BAL fluid were observed (Fig. 4A). Interestingly, only low-level reactivity for total IgG was seen in i.p. vaccinated animals, with no evidence of *Pseudomonas*-specific IgA (Fig. 4B). The lack of IgA in pulmonary secretions from i.p. vaccinated mice was not unexpected, since this antibody was similarly not observed in serum from these animals. Although these results clearly do not rule out a function of IgG-mediated protection in orally vaccinated mice, they do suggest that IgA may have an important role.

Antisera induced by oral vaccination mediate efficient opsonic killing of multiple *P. aeruginosa* serogroup O11 strains. Because we did not observe a significant level of protection against *P. aeruginosa* challenge in i.p. vaccinated animals, we

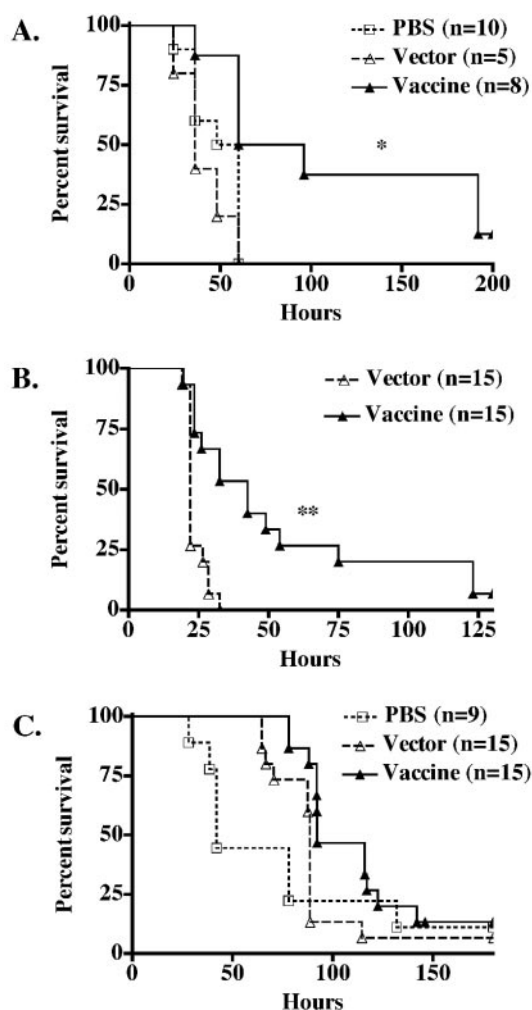


FIG. 3. Survival rates for orally and i.p. vaccinated animals after intranasal challenge with *P. aeruginosa* strain 9882-80. Animals immunized by oral (A and B) and i.p. (C) inoculations were challenged intranasally with *P. aeruginosa* strain 9882-80 (serogroup O11) and carefully monitored for survival. PBS- and vector-immunized mice served as controls. Infectious doses were calculated to be 1.12×10^8 CFU (A), 2.34×10^8 CFU (B), and 1.13×10^8 CFU (C). Results are represented as Kaplan-Meier survival curves, and differences in survival were calculated by the log-rank test. (A) In the first oral vaccination study, increased survival was observed only for mice that received the vaccine strain (single asterisk) (*P* values determined by log-rank analysis: for PBS versus vaccine, 0.0128; for vector versus vaccine, 0.0084; and for PBS versus vector, 0.2813). Median survival times were as follows: with PBS, 54 h; with vector, 36 h; and with vaccine, 78 h. (B) In the second oral vaccination study, increased survival was again seen for mice that received the vaccine strain (double asterisks) (*P* value for vaccine versus vector: 0.0002). Median survival times were as follows: with vector, 22 h; and with vaccine, 42.5 h. (C) No difference in survival was observed for mice that received the vaccine strain i.p. versus those that received control treatments (*P* values: for PBS versus vaccine, 0.135; for vector versus vaccine, 0.1178; and for PBS versus vector, 0.2341). Median survival times were as follows: with PBS, 42 h; with vector, 88.5 h; and with vaccine, 92.3 h. Values in parentheses indicate the numbers of animals used.

focused our analysis on the antisera induced by oral vaccination and measured the opsonic titers induced by the vaccine in opsonophagocytosis assays. The antisera mediated biologically significant killing (>50%) of all serogroup O11 strains tested

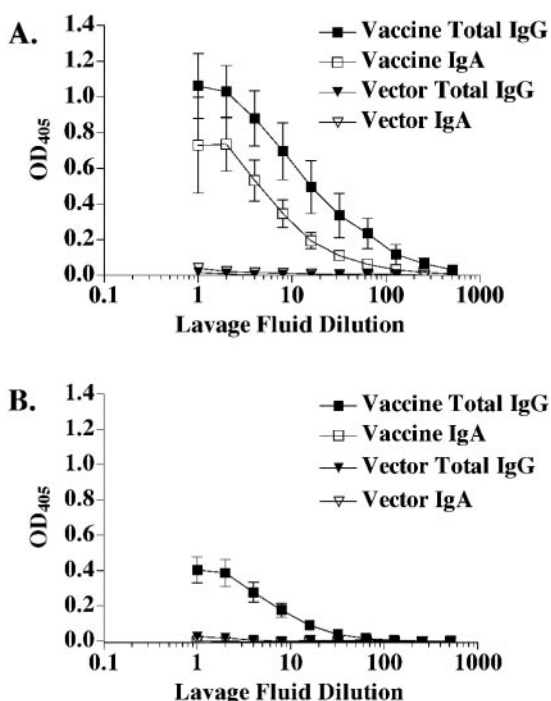


FIG. 4. BAL of orally and i.p. vaccinated mice. For lung lavages of orally (A) and i.p. (B) inoculated mice, animals were anesthetized and the tracheas were exposed by standard procedures and cannulated. A total of 2 ml of PBS was instilled into the lungs and carefully extracted. BAL fluid was analyzed by an ELISA on plates coated with PA103 whole organisms and probed for bound IgG and IgA. BAL fluid dilutions are plotted as log₁₀ units as a function of reactivity at OD₄₀₅. Error bars indicate standard deviations.

at serum dilutions of up to 1:200 (Fig. 5A). Consistent with the data obtained in the survival study with the heterologous serogroup O6 challenge strain, no killing of strain 6294 was seen, suggesting again that any cross-reactive antibodies that are present after oral immunization are not protective and do not mediate bacterial killing. Therefore, it is likely that increased survival is mediated through antibody-dependent bacterial clearance by opsonophagocytosis and that protection can be correlated with the heterologous serogroup O11 strains.

Effect of oral immunization on *Pseudomonas* clearance from the lungs. To determine whether the orally delivered vaccine promotes *Pseudomonas* pulmonary clearance and limits dissemination of the challenge strain to the blood, vaccinated mice were challenged intranasally with a sublethal dose of 9882-80 (1.2×10^7 CFU), and the lungs and spleens were removed for the determination of bacterial CFU at 6 and 12 h postinfection. At 6 h, no statistical difference was seen in bacterial loads in the lungs of vaccine-inoculated animals and control animals, but reductions in CFU of 8.3 and 24.1% were observed relative to the CFU seen in vector- and PBS-inoculated animals, respectively (Fig. 5B). In contrast, the numbers of viable bacteria in the lung tissues of vaccine-inoculated mice at 12 h postinfection were significantly lower than those seen in PBS- and vector-inoculated mice (ca. 68.4% decrease for vector versus vaccine and ca. 53.3% decrease for PBS versus vaccine) (Fig. 5B). No difference in bacterial CFU was observed between mice inoculated with PBS and mice inoculated

with vector ($P = 0.727$). In addition, the reduction in bacterial CFU in vaccine-inoculated mice achieved biological significance (>50% reduction) relative to the CFU seen in the lungs of vector- and PBS-inoculated mice. No bacteria were recovered from the spleens of any of the mice at either time point.

DISCUSSION

P. aeruginosa is usually associated with chronic pulmonary infections in cystic fibrosis patients, where it is a common cause of morbidity and eventual mortality in these individuals. However, *P. aeruginosa* is also a problematic infectious organism in the hospital environment, causing severe pneumonia in intensive care units and in ventilator-dependent patients. It is believed that the initial site of colonization is localized to the upper respiratory epithelium; therefore, inducing mucosal immunity to this pathogen appears to be an ideal strategy for the prevention of infection.

Vaccines that generate antibodies to the smooth-O-antigen portion of LPS have been shown to mediate the most effective immunity to *P. aeruginosa* (12, 38, 42, 48). The efficacy of these vaccines is primarily due to efficient opsonic killing that has been observed in vitro and thus serves as a strong correlate of protection (43).

Approximately 10 serogroups of *P. aeruginosa* are considered clinically relevant, but many subtype strains that harbor slight chemical modifications in the O unit exist within these designations. These differences give rise to variants within each serogroup that cause difficulties in making a broad-based vaccine. Polyvalent preparations of purified high-molecular-weight O polysaccharides, injected i.p., were found to be highly immunogenic in C3H/HeN mice but only elicited antibodies that facilitated opsonic killing of the strains used to make the vaccine (25). Further efforts to construct a divalent vaccine with high-molecular-weight O polysaccharides that individually were shown to stimulate the broadest opsonic capabilities among *P. aeruginosa* serogroup O2/O5 strains were proven to be unsuccessful and actually diminished immune effectiveness (27). One of the explanations suggested for these results was competition among related O antigens for the limited membrane immunoglobulin repertoire on B cells (27). However, the structural complexity that leads to drawbacks for vaccine development based on purified LPS O antigen may be resolved when this structure is expressed in the context of a live attenuated organism.

Priebe et al. demonstrated that intranasal administration of a live attenuated *P. aeruginosa* O2/O5 Δ aroA strain elicits high levels of opsonic anti-O-antigen titers to multiple serogroup O2/O5 strains (52, 53). A similar strategy of attenuation has been in use for *Salmonella* organisms for decades. Attenuated *Salmonella* strains have been reported to be effective vaccine carriers for both heterologous proteins (5, 20, 40, 41) and polysaccharides (45, 56, 63) because of their innate ability to activate the common mucosal immune system and systemic immunity after mucosal delivery. We have taken advantage of *Salmonella* as a vaccine vehicle for delivery of the *P. aeruginosa* serogroup O11 O antigen and evaluated protection in a pneumonia model. In the present study, we have shown that oral vaccination with SL3261 expressing the *P. aeruginosa* PA103 serogroup O11 O antigen provides increased survival to chal-

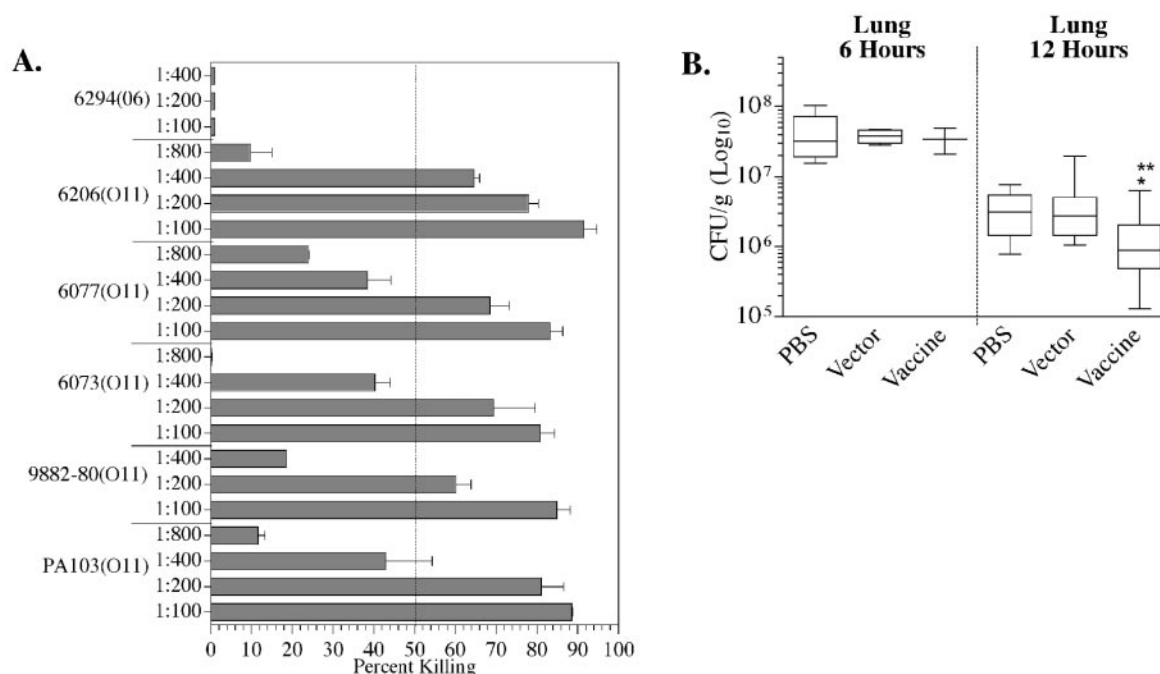


FIG. 5. Killing of *P. aeruginosa* organisms in opsonophagocytosis assays and clearance from mouse lungs at 6 and 12 h after infection with *P. aeruginosa* 9882-80. (A) Opsonophagocytosis assay of *P. aeruginosa* serogroup O11 strains and a heterologous serogroup O6 strain, 6294, with dilutions of pooled sera raised to the vaccine strain by oral vaccination of BALB/c mice. Shaded bars represent the mean percent killing, and error bars represent the standard deviation. Results were determined from two independent experiments performed in duplicate and were calculated relative to results obtained with a preimmune serum control. Biological significance was obtained at 50% killing. (B) Orally vaccinated mice received a nonlethal dose of *P. aeruginosa* 9882-80 (1.2×10^7 CFU), and lungs were removed, homogenized, and plated for identification of bacterial CFU at the indicated hours. Box and whisker plots show bacterial CFU in log₁₀ units. The box is marked by the median and depicts the first and third quartiles, while the whiskers depict the range. Differences in viable CFU were determined by one-way analysis of variance. No difference in viable CFU was seen among PBS, vector, and vaccine groups at 6 h postinfection ($P = 0.9613$). However, a significant decrease in CFU was observed at 12 h postinfection for vaccine-inoculated mice compared to PBS- and vector-treated mice (single asterisk) (P values: overall, for all data sets, 0.0424; for vector versus vaccine, 0.022; and for PBS versus vaccine, 0.043). Double asterisks indicate biologically significant data, i.e., 50% reduction of bacteria by vaccine versus controls.

lenge with *P. aeruginosa* in an acute pneumonia model. Oral vaccination with this strain was shown to stimulate broad serogroup O11-specific serum total IgG and IgA responses to multiple serogroup O11 strains (Fig. 1A to C). Analysis of BAL fluids from orally vaccinated mice also revealed the presence of *Pseudomonas*-specific IgG and IgA (Fig. 4A), suggesting that the gut-associated lymphoid tissue (GALT) is sufficiently activated by the vaccine construct for antibody-secreting B cells to localize to the respiratory lymphoid tissue.

Previous studies suggested that systemic immunity, from either subcutaneous vaccination (6) or i.p. vaccination (45) with *P. aeruginosa* vaccine constructs, was as effective as mucosal delivery of the vaccine in a mucosal challenge. To determine whether this notion held true in the *Pseudomonas* pneumonia model used here, we vaccinated mice by i.p. injection. Work by other groups has shown that i.p. administration of attenuated *Salmonella* organisms produces potent systemic antibody responses but not mucosal stimulation, as determined by the absence of an IgA response in serum and mucosal secretions (24, 62). As expected, i.p. inoculation with the vaccine strain generated potent *Pseudomonas*-specific serum IgG responses (Fig. 1B to D). We did not observe the presence of *Pseudomonas*-specific IgA in serum (data not shown) or BAL fluid (Fig. 4B) from i.p. vaccinated mice, consistent with previous

reports and suggesting that these mice lacked a mucosal response to the recombinant antigen. Furthermore, these results are comparable to those of a previous report that sought to analyze the response to *Salmonella* LPS after multiple routes of immunization with SL3261 in BALB/c mice (24). Unlike IgA, IgG was present in BAL fluid (Fig. 4B) from i.p. vaccinated animals, likely because of passive diffusion from the circulation.

Oral administration of the vaccine strain was shown to provide increased survival of pneumonia caused by *P. aeruginosa* after challenge doses that were 6 and 12 times the LD₅₀, compared to the results obtained with the PBS and vector controls. In contrast, i.p. administration of the vaccine strain did not confer the same level of protection in mice with a challenge dose that was six times the LD₅₀, compared to the results obtained with the vector control. This result was unexpected based on the significantly higher *Pseudomonas*-specific serum IgG titers that were observed in these animals compared to animals that received the oral vaccine (Fig. 1). Interestingly, mice that were i.p. vaccinated with the *Salmonella* vector survived about twice as long after infection as did mice orally immunized with the same construct. *Salmonella* i.p. vaccination may elicit cross-reactive antibodies to *P. aeruginosa* antigens that are not produced after oral immunization. Indeed, West-

ern analysis of *P. aeruginosa* whole-cell lysates probed with pooled sera from animals inoculated i.p. with the vector revealed cross-reactive proteins (data not shown). However, the same antisera did not promote the killing of *P. aeruginosa* strains in opsonophagocytosis assays (data not shown). Our data are in contrast to previous findings that systemic stimulation was sufficient to obtain significant protection at a mucosal challenge site. Cripps et al. demonstrated that the activation of GALT in rats with paraformaldehyde-killed *P. aeruginosa* leads to mucosal and systemic *P. aeruginosa*-specific antibody responses that promote pulmonary clearance in a live bacterial intratracheal challenge setting (6). In this previous study, vaccine administration was performed by mucosal routes (intra-Peyer's patch, intratracheal, oral-intratracheal, and oral) and systemic routes (subcutaneous); significant protection was observed with each route of immunization. However, this group concluded that mucosal administration leads to overall enhanced clearance from the lungs (6). In addition, Pier et al. reported that systemic vaccination with high-molecular-weight O polysaccharides was sufficient to induce systemic IgG and IgM titers that were shown to promote *P. aeruginosa* clearance from the gastrointestinal tract (45).

Previous reports supported antibody-dependent killing of *P. aeruginosa* in the lungs by identifying the role of pulmonary immune cells, namely, alveolar macrophages and polymorphonuclear leukocytes, involved in clearing *P. aeruginosa* from rat lungs after GALT stimulation (1, 2, 7, 8). Additionally, the results of the present study suggest that mucosal activation with our recombinant SL3261 vaccine (via the oral route) is more efficacious than systemic stimulation alone in protecting against *P. aeruginosa* in an acute pneumonia model.

We found that sera from orally vaccinated mice were reactive to five serogroup O11 clinical isolates. While helpful, serum reactivity does not correlate with protection from infection with these organisms. We performed opsonophagocytosis assays using pooled sera from orally vaccinated mice to identify the ability of antisera to facilitate the killing of each serogroup O11 strain. Efficient killing of all serogroup O11 strains was observed at serum dilutions as high as 1:200. Interestingly, >64% killing of strain 6206 was observed at a serum dilution of 1:400, even though the recombinant O antigen was derived from strain PA103 (Fig. 5A). These results differed from those of previous reports, where LPS polysaccharide vaccines were shown to stimulate poor opsonic titers against organisms that were within the same serogroup but that were not contained in the vaccine (11, 12, 14, 25, 27). Our results suggest that the expression of recombinant O antigens in a live vector may improve the production of opsonic antibodies to different strains within serogroup O11. Similar results were observed with O2/O5 subtype strains when a live attenuated *P. aeruginosa* O2/O5 vaccine was used (52). Work is currently under way in our laboratory to determine whether a cocktail vaccine consisting of multiple *Salmonella* organisms, each expressing a different *P. aeruginosa* O antigen, can provide the same level of protection against acute pneumonia caused by *P. aeruginosa*. Such work will help to elucidate whether competition among O antigens can be alleviated by a live-attenuated organism.

We also compared the levels of bacteria in mice orally inoculated with PBS, vector, and vaccine after a sublethal challenge with 9882-80 (Fig. 5B). This dose was chosen because it

was shown to be cleared by naive mice within 72 h, thus resulting in complete survival of the animals (data not shown). Therefore, we measured the levels of viable *Pseudomonas* at 6 and 12 h to determine whether oral vaccination enhanced the ability of mice to clear 9882-80 early during infection. At 6 h, only a low-level decrease in the bacterial load was observed in vaccine-inoculated animals compared to control animals. At 12 h, statistical as well as biological significance was achieved. Therefore, it appears that oral vaccination with the vaccine construct confers an enhanced ability to remove *P. aeruginosa* organisms from the lungs of infected animals. Altogether, these results suggest that increased survival is due to antibody-dependent opsonization of bacteria from the lungs of infected animals, a process that is mediated by the presence of *P. aeruginosa* O-antigen-specific antibodies. Work is currently under way to examine the roles of IgG and IgA in the *Pseudomonas* pneumonia model. These results could have major implications for susceptible individuals in the hospital environment, where the goal would be to prevent initial colonization with *P. aeruginosa*. Moreover, Goldberg et al. previously showed that the O11 construct is expressed in the attenuated human *Salmonella* serovar Typhi vaccine strain Ty21a (22), suggesting that our vaccine construct is potentially useful in humans.

In conclusion, we have shown that orally delivered recombinant attenuated *Salmonella* is a suitable vaccine vector for the induction of protective immune responses against pneumonia caused by *P. aeruginosa* in mice. Further work is needed to determine whether *Salmonella* can support the expression of other clinically relevant O antigens from *P. aeruginosa* for the construction of a cocktail vaccine. In a clinical setting, such a vaccine would be ideal for the delay or prevention of *P. aeruginosa* infection in susceptible individuals.

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